East-West Divide: temperature and land cover drive spatial variation of Toxoplasma gondii infection in Eurasian Otters (Lutra lutra) from England and Wales

Journal:	*Parasitology*
Manuscript ID	PAR-2016-0216.R2
Manuscript Type:	Research Article - Standard
Date Submitted by the Author:	04-May-2017
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Key Words:	Landscape ecology, Meteorological variation, Spatial distribution, Zoonosis, Toxoplasmosis, Otter

East-West Divide: temperature and land cover drive spatial variation of *Toxoplasma gondii* infection in Eurasian Otters (*Lutra lutra*) from England and Wales

Running title: Drivers of spatial variation in UK *Toxoplasma gondii*

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SUMMARY

Toxoplasma gondii, a zoonotic parasite of global importance, infects all endothermic vertebrates, with extensive health implications. The prevalence of this parasite is seldom monitored in wildlife. Here, a semi-aquatic species, the Eurasian otter (*Lutra lutra*) was used as a model to assess the potential effect of climate, land cover and biotic factors on *T. gondii* seroprevalence in British wildlife. The Sabin-Feldman cytoplasm modifying dye test identified *T. gondii* antibodies in 25.5% of blood samples from otters found dead, mainly as road-kill, in England and Wales, between 2004 and 2010. Otters in the east of England were more likely to be infected with *T. gondii* than those in western regions. Land cover and temperature are key determinants of *T. gondii* infection risk, with more infection in arable areas, and lower infection where temperatures are higher. The probability of *T. gondii* infection increased with host age, reflecting cumulative exposure with time, but there was no association between *T. gondii* seroprevalence and cause of host death.

KEYWORDS

1. Landscape ecology
2. Meteorological variation
3. Spatial distribution
4. Zoonosis
5. Toxoplasmosis
6. Otter

HIGHLIGHTS

1. *Toxoplasma gondii* prevalence in the Eurasian otter in England and Wales is 25.5%
2. *T. gondii* infections reduce with increased long-term average minimum temperatures
3. *T. gondii* prevalence in otters was higher in arable areas
4. Otters were more likely to be infected in the East than the West of the UK
5. No apparent link between *T. gondii* infection and cause of otter death

INTRODUCTION

The parasitic protozoan *Toxoplasma gondii* infects a wide range of hosts worldwide, including all endothermic vertebrates (Dubey and Beattie, 1988; Tenter *et al*., 2000; Hill and Dubey, 2002). Felids are the only known definitive host of *T. gondii* (see Miller *et al*., 1972; Dubey, 1998), and excrete environmentally-resistant oocysts in their faeces (Dubey *et al*., 2010). Zoonotic infection occurs following ingestion of sporulated oocysts from the environment, contaminated water or food (Fayer *et al*., 2004; Tenter *et al*., 2000), or ingestion of bradyzoites (tissue cysts) in meat (Dubey, 1998; Hill *et al*., 2006) but not fish (Zhang *et al*., 2014). *T. gondii* can also be spread congenitally (Hill *et al*., 2006), leading to ocular lesions (Couvreur and Desmonts, 1962) and, in some cases, miscarriage (Flatt and Shetty, 2013). The parasite is notorious because of its ability to manipulate host behaviour, resulting in increased predation of infected rodents by the definitive host (Webster, 2007; Hari Dass and Vyas, 2014). It is unclear whether infection with *T. gondii* changes specific behaviours in wildlife, but increased risk-taking behaviour may occur, with Hollings *et al*. (2013) finding that road-kill marsupials were more likely to be infected than those culled in control programmes.

Domestic cats can release on average 84 million oocysts up to a month after initial infection (Dubey and Beattie, 1988; Dabritz *et al*., 2007). Oocysts are the only environmentally infective stage of *T. gondii* and are resilient, resulting in local 'hotspots' of the transmissible stage in the environment (Fayer *et al*., 2004). Unsurprisingly, wildlife in areas with high cat density are subject to increased *T. gondii* infection risk (Hollings *et al*., 2013). Spatial variation in abiotic conditions is also likely to drive differences in the distribution of *T. gondii* oocysts, affecting host exposure. Resistance of oocysts to short periods of drying and freezing (Kuticic and Wikerhauser, 1994; Frenkel, 2000), due to the physiochemistry of their bilayered wall, enhances their survival (Dumètre *et al*., 2013). Sporulation of oocysts is inhibited below -6 °C (Dumètre and Dardé, 2003), but at 25 °C they

remain viable in water for over 200 days (Dubey, 1998). Generally infection of wildlife is associated with mild, moist environments experiencing infrequent periods of freezing (Dubey and Beattie, 1988; Afonso *et al*., 2013; Sevila *et al*., 2014).

Inter-annual variation in *T. gondii* infection is associated with climatic variation; very dry, hot summers or very cold winters result in low oocyst survival, thus reducing the risk of infection (Tizard *et al*., 1976; Simon *et al*., 2011; Gilot-Fromont *et al*., 2012; Gotteland et al., 2014). High seroprevalence is associated with high farm densities and high numbers of European wild and domestic cats (Afonso *et al*., 2013; Gotteland *et al*., 2014). Agricultural practices may facilitate parasite transmission between domestic livestock and wildlife (Rosenthal, 2009) due to irrigation of soils and soil disturbance by livestock, which increases parasite survival and distribution (Lehmann *et al*., 2003). It seems intuitive that oocysts, which can survive for over a year in the soil (Frenkel and Dubey, 1973), will eventually be washed into freshwater and marine habitats by run-off from land (Fayer *et al*., 2004; Dabritz *et al*., 2007; Jones and Dubey, 2010). There is some evidence for *T. gondii* infection in marine animals (example: sea otters, Cole *et al*., 2000; striped dolphins, Di Guardo *et al*., 2010; and British marine mammals, Forman *et al*., 2009). Despite this, little research has been undertaken on freshwater systems and how land cover affects the risk of infection.

Eurasian otters (*Lutra lutra)* have a widespread distribution, covering parts of Europe, Asia and Africa (Corbett, 1966). Wild otters that utilize freshwater, marine and terrestrial habitats can be considered a sentinel for naturally acquired *T. gondii* infection (Chadwick *et al*., 2013). The aim of the current study was to investigate whether *T. gondii* seroprevalence in otters is associated with abiotic (meteorological factors and land cover) and biotic (host age, sex and cause of death) factors. Specifically, it is hypothesised that higher infection levels in otters will be evident in: (1) areas with mild temperatures, because the viability of oocysts in the environment will be prolonged; (2) areas

dominated by arable land, due to increased oocyst dispersal; and (3) road-killed animals compared to those dying from natural causes due to increased risk taking behaviour.

MATERIALS AND METHODS

Sample collection

Eurasian otters (88.9% road-kill) reported in England and Wales by members of the public were collected by environmental organisations and sent to the national monitoring programme at Cardiff University Otter Project along with location data. Grid references, maps and site descriptions are supplied, and are cross referenced to validate locations. Carcasses were stored at -18 °C and thawed 48 h prior to necropsy (see Simpson 2000). In total, the current study analysed data from 659 otter cadavers collected 2004-2010, including 271 samples analysed previously by Chadwick *et al*. (2013). Blood samples were collected from the thoracic cavity during necropsy by submerging a 1.5 ml eppendorf in the pooled (unclotted) blood, and stored at -18 °C prior to analysis.

Sabin-Feldman cytoplasm modifying dye test for detection of T. gondii antibodies

Blood samples were defrosted, centrifuged and the Sabin-Feldman cytoplasm modifying dye test (Sabin and Feldman, 1948) applied to detect *T. gondii* antibodies, at the Public Health Wales *Toxoplasma* Reference Unit, Swansea. In brief, live *T. gondii* and accessory factor (human seronegative serum samples) were added to serial dilutions of the otter blood samples and incubated at 36-38 °C for 60 min, to encourage complement-mediated killing of *T. gondii*. Methylene blue was then added for 5 min. Living cells, which took up the dye, and unstained cells, were identified using an inverted microscope (Leitz Diavert, ×32 objective and ×40 eyepiece magnification). The end point titre of each serum sample was determined when ca. 50% unstained (dead) *T. gondii* cells

were counted in a serial dilution. When the dyed cells were difficult to identify or showed a prozone phenomenon (a false negative due to high titres; Dzbenski and Zielinski, 1976), the test was repeated. A titre of 1/8, ≥4 international units/ml compared with the WHO international *Toxoplasma* control serum containing 1000 international units/ml, was considered indicative of infection. For one sample, the Sabin-Feldman dye test result was ambiguous and this was removed from the dataset. Here, prevalence refers to the percentage of seropositive hosts and, therefore, includes current and/or past infection in individuals.

Climate and land cover

The distribution of the 659 otter mortality sites was plotted using ArcMap GIS (version 9.2) and each location assigned to one of eight regions based on groups of river catchments (Fig. 1). Otters have home ranges up to 40 km (Kruuk, 2006). In order to estimate climate and land cover at a scale appropriate to otter range, ArcMap GIS was used to collate data from within a circular area 20 km in radius, centred on each otter mortality location (after Chadwick *et al*., 2011).

Long-term average climatic data (1981-2006) from UK climate projections were used to map spatial variation in climate (UKCIP09; Perry and Hollis, 2005) specifically: average minimum temperature (°C), average days of ground frost and average rainfall (mm), at a 5 km^2 resolution. These meteorological variables were selected as they are known to affect survival of oocysts in the environment (Dubey and Beattie, 1988; Dubey, 1998; Dumètre and Dardé, 2003). For climatic variables, the mean value was calculated within each 20 km radius area.

Data from the Countryside Information Services (www.ceh.ac.uk/products/software/cehsoftware-cis.htm) were used to map percentage cover of arable land, broadleaf woodland, coniferous woodland, improved grassland, semi-natural grassland, upland and built-up areas, at a 1 km^2

resolution, based on digital spatial data licensed from the Centre for Ecology & Hydrology, NERC (CEH Land cover 2000; Fuller *et al*., 2002). For land cover, if an otter ranging region (20 km radius) had one land cover type >50% of the area this was nominated as the dominant land cover; if no single land cover formed >50% of the area, the area was classified as mixed. Other potentially important environmental characteristics were omitted due to data deficiency (cat density) or high levels of spatiotemporal variation (in-stream river characteristics).

Biotic associations

A range of data were collected at post-mortem, including: age-class (juvenile, sub-adult, adult), sex, cause of death, body length and body weight. Five individuals from the study group could not be sexed due to extensive damage to the carcass. Although month and year of death were collected they were not used in statistical modelling due to uncertainties regarding time of infection and date of death.

Cause of death was categorised as road traffic accident (RTA) or non-RTA. Further subdivisions of the latter were considered (namely bite wounds, blow to head, drowned, emaciated, infection, snared, shot); but small samples sizes precluded more detailed analysis. Size and reproductive indicators were used to categorise otters by age-class, as juvenile (females <2.1 kg, males <3 kg), sub-adult (females ≥2.1 kg with no sign of reproductive activity, males ≥3 kg with a baculum <60 mm in length) or adult (females with signs of reproductive activity, males with baculum ≥60 mm).

Statistical Analyses

All statistical analyses were performed in R (version 3.2.3; R development Core Team, 2015). A generalised linear model with a binomial error distribution was fitted to the *T. gondii* prevalence data, to examine the probability of *T. gondii* infection of otters using meteorological data (25-year

mean annual: minimum temperature, ground frost days, rainfall), land cover type (arable land, semi-natural grassland, improved grassland and mixed), biotic data (otter age-class, length and sex), cause of death and region, as explanatory variables. The interaction term Sex:Age was also included in order to test whether age differences varied with sex or vice versa. All terms were included in the original model (AIC_i) with one term removed at a time (AIC_b), using the drop1 function in R, which employs the Akaike Information Criterion (AIC) method to identify the best fitting and most efficient model (Thomas *et al.*, 2013). Variables were excluded from the final model when the difference between AIC_b and AIC_i was greater than two (Thomas *et al.*, 2013). The final model used average minimum temperature, land cover, age and sex to explain variation in the probability of an otter being infected with *T. gondii*. The distribution of deviance residuals was examined to check for lack of fit. Other typical model checking procedures (such as overdispersion) are not valid for Bernoulli GLMs (Thomas *et al.*, 2013). Additionally, a Pearson's Chi-squared test was performed to identify any differences between the seroprevalence of otters killed in road traffic accidents (RTA) and any other cause of death (non-RTA).

Spatial analysis (SaTScan, version 9.1.1; Bernoulli model) was used to identify clustering between *T. gondii* prevalence in otters and the location and time that the otter was found dead. SaTScan employs centroids that are distributed across the region of interest (England and Wales), to compare the observed number of cases (*T. gondii* positive otters) to the expected number of cases, if they were randomly distributed, using a likelihood ratio test (Kulldorff, 1997, Kulldorff *et al.*, 1998). In the absence of knowledge on specific otter territories and to provide a sufficient scale, the mean x and y NGR coordinates for the counties in England and Wales were used to describe the centroids for analysis.

RESULTS

Toxoplasma gondii antibodies were present in 25.5 % (168/659) of otters, with infections widely distributed across England and Wales (Fig. 1). Both abiotic and biotic variables explained significant varation in the prevalence of *T. gondii* (Table 1)

Climate and land cover

There was a negative association between annual minimum temperature and *T. gondii* infection status ($z_{1,646} = -3.88$, $p \leq 0.001$, where z is the test statistic [in this case the Wald statistic, which is the regression coefficient divided by its standard error]), such that probability of infection reduces with increased average minimum temperature (Fig. 3). In areas with primarily arable land, primarily the East, otters were more likely to be seropositive than in areas dominated by improved grassland ($z_{3,646} = 2.35$, $p = 0.019$) or semi-natural grassland ($z_{3,646} = 1.99$, $p = 0.047$). Although marginally non-significant, otters were less likely to be infected with *T. gondii* in areas with mixed land cover, than those found in areas with predominantly arable land ($z_{3,646} = 1.95$, $p = 0.052$). There was no significant difference between improved grassland, semi-natural grassland or mixed land cover ($p > 0.05$). Although the interaction term temperature: landcover was non-significant, model predictions suggest that where average minimum temperatures were high (8 ^{o}C), the probability of infection was low across all land covers, whereas at low minimum temperatures (4 ^{o}C), probability differed between land covers, with probabilities in Arable > Mixed > Improved > Semi-natural. Where sex, age and temperature are controlled in the model to predict probabilities for male otters, at an average minimum temperature of 6 ^{o}C, the relative probabilities of seropositivity is 0.426 ± 0.051 in arable land, compared to 0.252 ± 0.030 in mixed, 0.189 ± 0.038 improved grassland and 0.116 ± 0.042 semi-natural land (Fig. 3). There was no significant association of *T. gondii* prevalence with number of ground frost days and rainfall ($p > 0.05$).

There was no significant clustering, either spatially or temporally. Although seroprevalence was higher in the North East, Anglian and Southern Regions than the Welsh, North West and South West Region (Fig 1), model outputs indicate no significant differences between regions, suggesting that climate and land cover differences adequately explain regional variation.

Biotic associations

Seroprevalence increased with age; juveniles (8%; N = 25); sub-adults (23.3%; N = 271) and adults (28.7%; N = 358; Fig. 2; p =0.021). There was a significant difference in seroprevalence of *T. gondii* between the sexes; females were more likely to be infected than males (difference in probability of infection = 0.4 ± 0.2; $z_{1,646}$ = 2.02, p = 0.044). There was no significant age:sex interaction, i.e. the effect of age did not differ between the sexes, and no significant effect of length or cause of death.

DISCUSSION

This study examined the seroprevalence of *Toxoplasma gondii* in the Eurasian otter (*Lutra lutra*) in relation to climate, land cover and biotic variables across England and Wales. It is the only study to have examined such associations in a semi-aquatic species, which might be considered at particular risk from infection, due to exposure to oocysts both on land, and oocysts accumulating and dispersed in water systems. Dispersal of oocysts in water might be expected to confound spatial variation of the parasite, particularly in aquatic or semi-aquatic hosts. Despite this, the current study shows that spatial variation in *T. gondii* distribution can be explained by average annual minimum temperature and land cover (see Gotteland *et al.*, 2014).

Cold climates have been linked with decreased seroprevalence of *T. gondii*, due to reduced oocyst

viability and risk of infection (Dubey *et al*., 1970; Frenkel and Dubey, 1973; Dumètre and Dardé, 2003). In the UK, this may explain low *T. gondii* seroprevalence in humans from Scotland (Food Standard Agency, 2012). The current study excluded Scotland however, due to lack of samples, and showed no association between days of ground frost and seroprevalence. This could be because minimum temperatures in England and Wales are not low enough to significantly reduce viability. Conversely, we found a negative association between temperature and seroprevalence, such that areas with higher temperatures had a lower probability of infection (contradicting hypothesis 1). This may reflect a reduction in viability due to high summer temperatures, as suggested by Gilot-Fromont *et al.* (2012).

Areas of arable land (primarily in the East of England) had relatively high seroprevalence (see also Chadwick *et al*., 2013) supporting hypothesis 2. Arable land in the UK is primarily in areas with relatively low rainfall, which is partially alleviated through irrigation (Environment Agency, 2009). Surface run-off tends to be high in arable areas, due to a combination of land drainage, low levels of soil organic matter and altered soil structure (Environment Agency, 2009). This may increase the number of oocysts being washed into water, potentially increasing the infection risk to otters. A link to high surface run-off is supported by Shapiro *et al*. (2010); they used surrogate *T. gondii* oocysts (autofluorescent, carboxylate-modified polystyrene microspheres) to show that after a period of dry weather, the first heavy rainfall which caused the ground to become saturated led to overland run-off 'flushing' oocysts from land to freshwater and into the ocean. Increased seroprevalence in arable areas might also reflect a correlation between land-use and cat density (e.g. related to high numbers of farm cats around grain stores), but there are insufficient data on either domestic or feral cat numbers in the UK to test this hypothesis.

T. gondii is notorious for its role as a host manipulator, with infected rodents and even primates becoming more risk-taking and active (Webster, 2007; Poirotte *et al*., 2016). In humans, *T. gondii*

infection has been associated with increased suicide attempts (Pederson *et al*., 2012) and increased likelihood of being involved in a road traffic accident (Flegr *et al*., 2009). More recently, though, Sugden *et al*. (2016) argue there is limited evidence that *T. gondii* in humans is related to poor impulse control, increased risk of personality aberrations or neurological impairment. For wildlife, it is difficult to quantify 'risky' behaviour, specifically whether road crossing is a perceived risk for an otter. More generally, regardless of infection status, there are behavioural traits associated with wildlife and road-crossing. For example, badgers are less likely to cross roads where there are high volumes of traffic (Clarke *et al*., 1998) and smaller mammals tend to avoid roads (McGregor *et al*., 2008). In the current study, cause of death was not associated with *T. gondii* seroprevalence (contradicting hypothesis 3). In contrast, Hollings *et al*. (2013) found significantly higher seroprevalence in road-kill compared to culled animals. Possibly, our analysis was limited by the relatively small sample size of non-road kill samples (11 infected and 47 uninfected individuals) and wide variation in cause of death within our non-RTA group.

The current study shows that the seroprevalence of *T. gondii* in the Eurasian otter (25.5%, 168/659) was lower than previously reported for this host (39.5%, 108/271, Chadwick *et al*., 2013; 100%, 6/6, Sobrino *et al*., 2007): probably a reflection of our increased statistical power with the larger sample size. The method used to identify the presence of antibodies determines whether an individual has become infected during its lifetime (e.g. Sobrino *et al*., 2007; Richomme *et al*., 2010). *T. gondii* seroprevalence in otters increased with age, presumably a reflection of cumulative exposure to *T. gondii* with time, and corroborates the findings of previous research (wild carnivores, Sobrino *et al*., 2007; mink, Sepulveda *et al*., 2011; otters, Chadwick *et al*., 2013; and wild boar, Richomme *et al*., 2010). Higher seroprevalence in females contrasts with previous reports which found no significant difference with sex (Eurasian otters, Sobrino *et al*., 2007; mink, Sepulveda *et al*., 2011) and is surprising, given both the larger home range of males (Kruuk, 2006; potentially increasing exposure risk), and a general trend toward greater male susceptibility to infectious

diseases (e.g. Zuk and McKean, 1996; Stoehr and Kokko, 2006). In cats, prey composition influences *T. gondii* infection risk (Afonso *et al*., 2007). Otters are largely piscivorous but do occasionally take mammals or birds (e.g. Blanco-Garrido *et al*., 2008). Variations in land-use, climate and geographical location may impact on the availability or preference for particular prey, affecting the risk of acquiring the infection via tissues cysts. Sexual differentiation in otter diet, combined with spatial variation in prey availability, may contribute to sex and spatial differences in risk of infection.

This study concludes that *T. gondii* seroprevalence in the Eurasian otter was associated with climatic, land cover and biotic factors in England and Wales. Probability of infection was extremely low in warmer areas, across habitats, perhaps relating to low summer survival of oocysts. The highest risk of infection was in arable areas, which may reflect greater oocyst transport with run-off. Developing our understanding of spatial variation in infection risk and its' drivers has clear implications for exposure risk in other species, including humans.

ACKNOWLEDGEMENTS

Members of the public reported otter carcasses, and collection coordinated by the Environment Agency (EA), UK. Cardiff University Otter Project was funded by the Environment Agency and Natural Resources Wales, with additional contributions made by the Somerset Otter Group. We appreciate the help of three anonymous reviewers as these helped improve the manuscript.

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532 **Zuk, M. and McKean, K.A.** (1996). Sex differences in parasite infections: patterns and processes.

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534 **Table 1:** Variables explaining *Toxoplasma gondii* seroprevalence in Eurasian otter (*Lutra lutra*).

535

536 **Variable**	**Degrees of freedom**	**LRT**	**P value**
538 Age	2	7.072	0.029
539 Sex	1	4.034	0.045
540 Land cover	3	14.197	0.003
541 Minimum temperature	1	16.096	≤ 0.001

542 **Figure 1: A)** Seroprevalence of *Toxoplasma gondii* in Eurasian otters (*Lutra lutra*) from England
543 and Wales. Seropositive otters are shown as black circles and seronegative otters as white circles.
544 The percentage of *T. gondii* seropositive otters is indicated for each of eight Regions (N=659); **B)**
545 Long-term average minimum temperature data (°C; 1981-2006) from UK climate projections
546 (UKCIP09); **C)** Land cover for England and Wales based on digital spatial data licensed from the
547 Centre for Ecology & Hydrology, © NERC (CEH Land cover 2000; Fuller et al., 2002) i - Broad-
548 leaved/mixed woodland; ii - Coniferous woodland; iii - Arable and horticulture; iv - Improved
549 grassland; v - Semi-natural grassland; vi - Mountain, heath and bog; vii - Built up areas and
550 gardens; viii - Standing open water.
551
552 **Figure 2:** *Toxoplasma gondii* seroprevalence in Eurasian otters (*Lutra lutra*) from England and
553 Wales. The percentage of *T.* gondii seropositive otters within each age-class for both males (dark
554 bars) and females (shaded bars). Five individuals could not be sexed due to the extent of their
555 injuries and were removed. Numbers of seropositive/total number of individuals in each group are
556 shown in parentheses.
557
558 **Figure 3:** Model predictions to show the probability of a *Toxoplasma gondii* infection in adult,
559 male Eurasian otters (*Lutra lutra*) for different land uses (arable, mixed, improved grassland and
560 semi-natural) as a function of average minimum temperature (^{o}C).
561

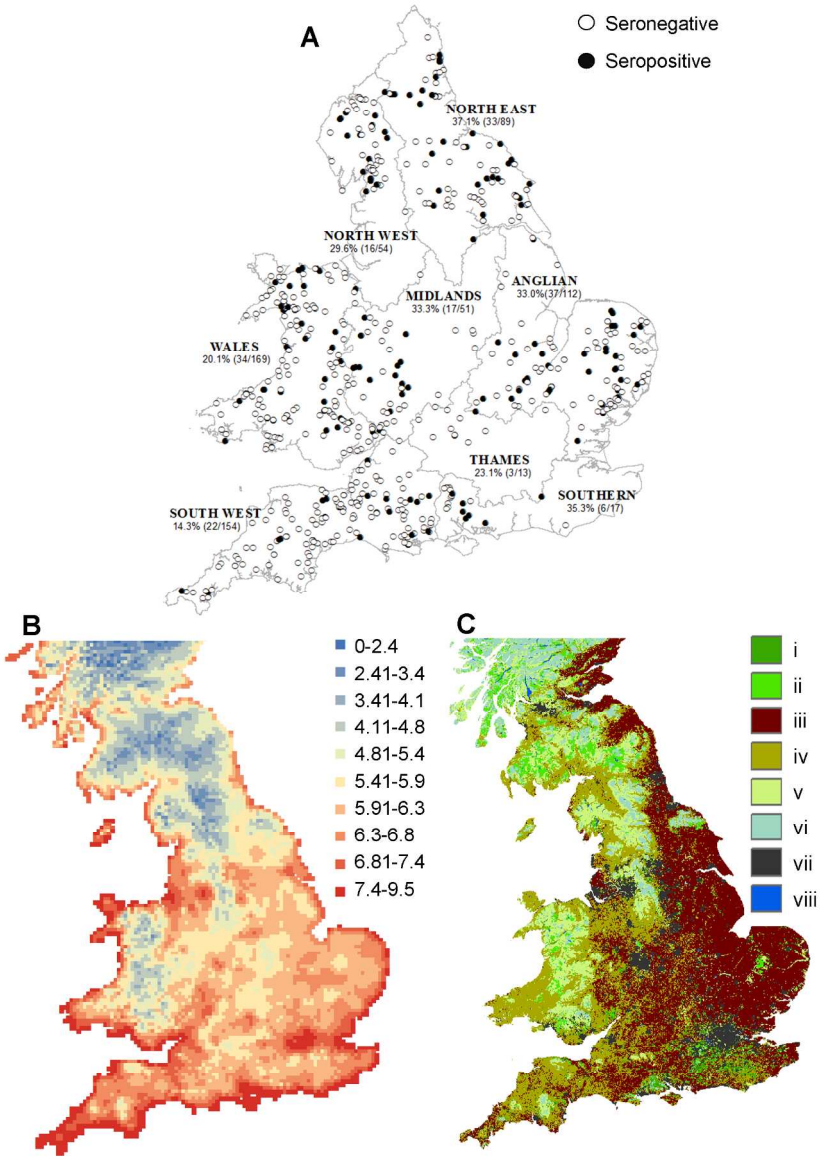

Figure 1: A) Seroprevalence of Toxoplasma gondii in Eurasian otters (Lutra lutra) from England and Wales. Seropositive otters are shown as black circles and seronegative otters as white circles. The percentage of T. gondii seropositive otters is indicated for each of eight Regions (N=659); B) Long-term average minimum temperature data (°C; 1981-2006) from UK climate projections (UKCIP09); C) Land cover for England and Wales based on digital spatial data licensed from the Centre for Ecology & Hydrology, © NERC (CEH Land cover 2000; Fuller et al., 2002) i - Broad-leaved/mixed woodland; ii - Coniferous woodland; iii - Arable and horticulture; iv - Improved grassland; v - Semi-natural grassland; vi - Mountain, heath and bog; vii - Built up areas and gardens; viii - Standing open water.

172x242mm (300 x 300 DPI)

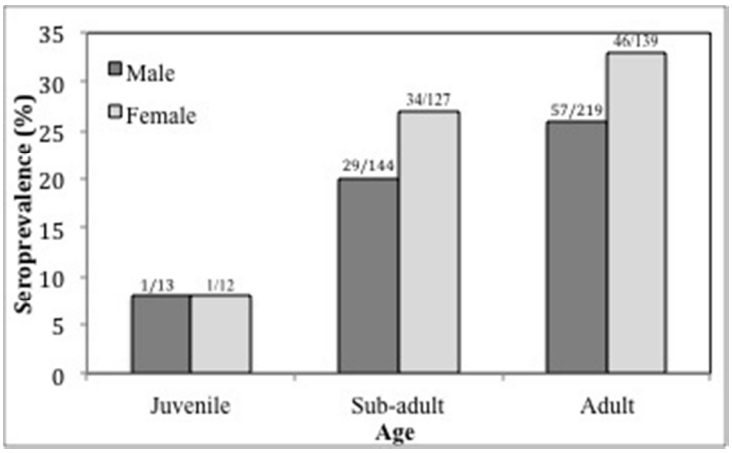

Figure 2: Toxoplasma gondii seroprevalence in Eurasian otters (Lutra lutra) from England and Wales. The percentage of T. gondii seropositive otters within each age-class for both males (dark bars) and females (shaded bars). Five individuals could not be sexed due to the extent of their injuries and were removed. Numbers of seropositive/total number of individuals in each group are shown in parentheses.

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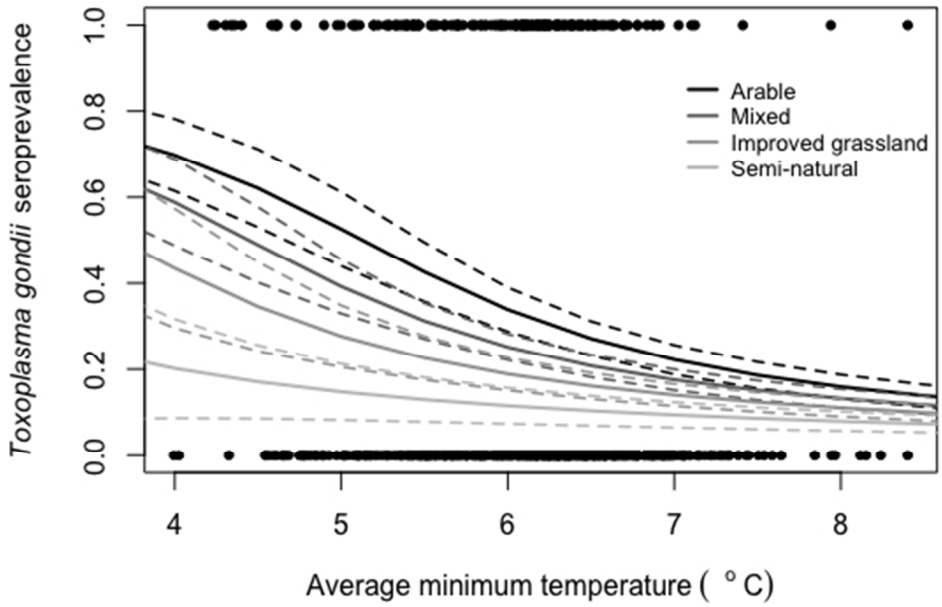

Figure 3: Model predictions to show the probability of a Toxoplasma gondii infection in adult, male Eurasian otters (Lutra lutra) for different land uses (arable, mixed, improved grassland and semi-natural) as a function of average minimum temperature (oC).

201x155mm (72 x 72 DPI)